(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 March 2002 (14.03.2002)

PCT

(10) International Publication Number
WO 02/20568 A2

(51) International Patent Classification[7]: C07K 14/00

(21) International Application Number: PCT/US01/27410

(22) International Filing Date:
4 September 2001 (04.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/656,399 6 September 2000 (06.09.2000) US

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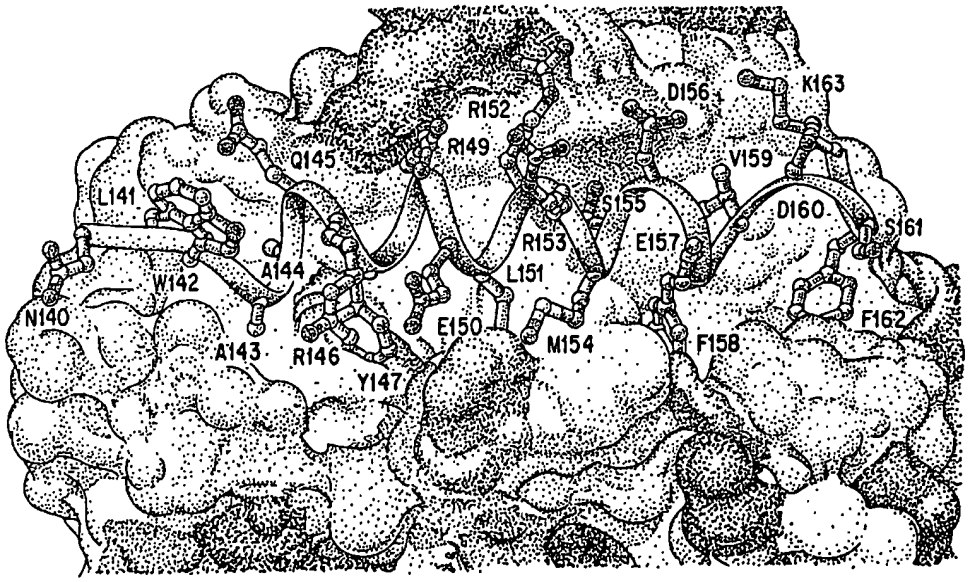
(81) Designated States *(national)*: CA, JP, MX.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MUTANT PEPTIDES DERIVED FROM BAD AND THEIR USE TO IDENTIFY SUBSTANCES WHICH BIND TO A MEMBER OF THE BCL-2 FAMILY OF PROTEINS



(57) Abstract: The present invention relates to peptides derived from the wild-type human Bad peptide which bind to a member of the Bcl-2 family of proteins. The peptides of the present invention can be used in an assay to identify candidate substances which induce or promote apoptosis in cells.

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MUTANT PEPTIDES DERIVED FROM BAD AND THEIR USE TO IDENTIFY SUBSTANCES WHICH BIND TO A MEMBER OF THE BCL-2 FAMILY OF PROTEINS

Technical Field of The Invention

The present invention relates to peptides derived from the wild-type human Bad peptide which bind to a member of the Bcl-2 family of proteins. The peptides of the present invention can be used in an assay to identify substances which bind to a member of the Bcl-2 family of proteins.

Background of the Invention

Apoptosis or programmed cell death (hereinafter "PCD") is a highly conserved and essential feature of development and homeostasis in higher organisms. Kelekar, et al., *Molecular and Cellular Biology*, 17(12):7040-7046 (1997). Apoptosis is a mechanism by which the body replaces older cells with new healthy cells, or by which a cell destroys itself to prevent the transmission of genetic errors to its progeny. In some cancers, for example, it is generally accepted that an alteration in the cell growth and/or cell death is due to the accumulation of several mutations in "key" genes which regulate these processes. The normal system is unable to eliminate cells containing these mutated genes and uncontrolled cell growth results. Thus, the aberrant nature of cell growth or apoptosis observed in cancer and other diseases is the consequence of malfunctioning of the regulatory pathways which control the equilibrium between cell growth and cell death.

One group of molecules that are involved in promoting or suppressing apoptotic responses is the Bcl-2 family of proteins. The Bcl-2 family contains proteins which either promote or inhibit cell death. Some of the inhibitors, frequently referred to as anti-apoptotic proteins, include: Bcl-2, Bxl-x_L, Mcl-1, adenovirus E1B 19K, Epstein-Barr virus BHRF1, and *Caenorhabditis elegans* Ced-9. Promoters of cell death, frequently referred to as pro-apoptotic proteins, include, for

example: Bax, Bak, Bad, Bik, Bid and Bcl-x_s. An important feature of the Bcl-2 family is that its members can interact (i.e. dimerize) with themselves or with other members of the family. (Kelekar, *supra*. Also see Ottilie, S., et al., *J. of Biol. Chem.*, 272(49):30866-30872 (1997)). It is believed that these protein-protein interactions are critically important in determining a cell's response to a death signal. (Kelekar, *supra*, also see Yang et al., *Cell*, 80:285-291 (1995)).

The three-dimensional structure of the Bcl-2 family member, Bcl-x_L has been elucidated (Muchmore et al., *Nature*, 381:335-341 (1996)). In its monomeric form, Bcl-x_L contains two central hydrophobic helices surrounded by amphipathic helices. A hydrophobic binding pocket, is created by the spatial proximity of three particular domains, known as BH1, BH2, and BH3, and provides a site for the death-promoting proteins to bind. Sattler M., et al., *Science*, 275:983-986 (1997). It also is known that the death-promoting proteins interact with this binding site through their own BH3 domains. *Id.* However, until the present invention, the precise nature of the protein-protein interactions between Bcl- x_L and the BAD protein of the Bcl-2 family were unknown.

It is known in the art that over-expression of the anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, which are often present in cancerous and other diseased cells, results in the blocking of apoptotic signals and allows said cells to proliferate. For example, high levels of Bcl-2 gene expression are found in a wide variety of human cancers. Furthermore, it is believed that by blocking Bcl-2 and Bcl-x_L, apoptosis can be induced in diseased cells, and can provide an effective therapy for cancer and other diseases caused by the impairment of the apoptotic process. Thereupon, there is a need in the art for an assay, which can be used to identify compounds which trigger or induce apoptosis by inhibiting the interactions between the Bcl-2 family of proteins.

Summary of the Invention

The present invention relates to an isolated and purified peptide derived from a wild-type human Bad peptide which binds to a member of the Bcl-2 family of proteins. This protein has the amino acid sequence:

$(Xaa)_n$-Tyr-$(Xaa)_3$-Leu-$(Xaa)_4$-Asp-Xaa-Phe-Val-$(Xaa)_n$

where each n independently has a value from 1 to 10 and where at least one of the Xaa amino acids is different from that of the wild-type human Bad peptide. Examples of a Bcl-2 family member include, but are not limited to, Bcl-x_L and Bcl-2.

The present invention also relates to a peptide selected from the group consisting of:

AAAAAQRYGRELRRMSDEFVDSFKK (SEQ ID. NO:1),
AAAAAQRYGRELRRMSDAFVDSFKK (SEQ ID. NO:2),
AAAAAQRYGRELRRMSDLFVDSFKK (SEQ ID. NO:3),
AAAAAQRYGRELRRMSDMFVDSFKK (SEQ ID. NO:4),
AAAAAQRYGRELRRMSDEFVDSKK (SEQ ID. NO:5),
AAAAAQRYGRELRRMSDKFVDSFKK (SEQ ID. NO:6),
NLWAAQRYGRELRRMSDKFVD (SEQ ID. NO:7),
AAAAAQRYGRELRRMSDEFVR (SEQ ID. NO:8), and
AAQRYGRELRRMSDEFVR ((SEQ ID. NO:9).

The present invention also relates to an assay for identifying compounds which bind to a member of the Bcl-2 family of proteins. The first step of the assay involves providing a candidate compound to be tested. The second step involves forming a reaction mixture by contacting the candidate compound with a Bcl-2 family protein and a labeled wild-type human Bad peptide or a labeled mutant peptide derived therefrom, having the amino acid sequence:

$(Xaa)_n$-Tyr-$(Xaa)_3$-Leu-$(Xaa)_4$-Asp-Xaa-Phe-Val-$(Xaa)_n$

where each n independently has a value from 1 to 10. Preferred peptides are mutants derived from wild-type Bad wherein said mutants have at least one Xaa amino acid that is different from that of the wild-type human Bad peptide. Most preferred mutant peptides have only one Xaa that is different from that of the wild-type human Bad peptide. The peptide can be labeled with

radioactive moieties, fluorescent moieites, enzymes, specific binding molecules or particles. Preferably, the peptide is labeled with a fluorescein compound, most preferably, fluorescein isothiocyanate or 5-carboxy-fluorescein. The third step involves incubating the reaction mixture under conditions sufficient to allow the candidate compound to bind to the Bcl-2 family protein. The final step involves determining whether the candidate compound has bound to the Bcl-2 family protein by determining whether the labeled Bad peptide has been displaced from binding to the Bcl-2 family protein.

Brief Description of the Figures

Figure 1 shows the NMR-derived structure of the wild-type Bad peptide bound to Bcl-x_L (surface representation). As the figure shows, Y147, L151, D156, F158, and V159 represent key contact points (amino acid residues Tyr, Leu, Asp, Phe, and Val, respectively) between the wild-type Bad peptide and the Bcl-x_L protein.

Figure 2 shows representative labeled peptides of the present invention.

Figure 3 shows six (6) synthetic compounds used in a competitive fluorescence polarization assay with the mutant peptides of the present invention.

Detailed Description of the Invention

I. The Present Invention

The present invention relates to peptides derived from the wild-type human Bad peptide which bind to a member of the Bcl-2 family of proteins. The peptides of the present invention can be used in an assay to identify substances which bind to a member of the Bcl-2 family of proteins.

II. Sequence Listing

The present application also contains a sequence listing. For the nucleotide sequences, the base pairs are represented by the following base codes:

Symbol	Meaning

A	A; adenine
C	C; cytosine
G	G; guanine
T	T; thymine
U	U; uracil
M	A or C
R	A or G
W	A or T/U
S	C or G

Symbol	Meaning
Y	C or T/U
K	G or T/U
V	A or C or G; not T/U
H	A or C or T/U; not G
D	A or G or T/U; not C
B	C or G or T/U; not A
N	(A or C or G or T/U)

The amino acids shown in the application are in the L-form and are represented by the following amino acid-three letter abbreviations:

Abbreviation	Amino acid name
Ala	L-Alanine
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartic Acid
Asx	L-Aspartic Acid or Asparagine
Cys	L-Cysteine
Glu	L-Glutamic Acid
Gln	L-Glutamine
Glx	L-Glutamine or Glutamic Acid
Gly	L-Glycine
His	L-Histidine
Ile	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Met	L-Methionine
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serine

Thr	L-Threonine
Trp	L-Tryptophan
Tyr	L-Tyrosine
Val	L-Valine
Xaa	L-Unknown or other

III. Peptides of the Present Invention

The present invention relates to mutant peptides derived from the BH3 domain of the naturally occurring or wild-type, pro-apototic human Bad peptide. As used herein, the term "naturally occurring" or "wild-type" human Bad peptide refers to the peptide shown in SEQ ID NO:10 having 25 amino acid residues and which is also disclosed in Ottilie, et al., *J. Bio. Chem.*, 272:30866-30872 (1997). The mutant peptides of the present invention can contain from about 15 to about 33 amino acid residues, preferably from about 18 to about 30 amino acids residues, and most preferably about 25 amino acids residues. Specifically, the mutant peptides of the present invention have the following amino acid sequence:

$$(Xaa)_n\text{-Tyr-}(Xaa)_3\text{-Leu-}(Xaa)_4\text{-Asp-Xaa-Phe-Val-}(Xaa)_n$$

where each n independently has a value of from 1 to 10 and where at least one of the Xaa amino acids is different from that of the wild-type Bad peptide.

Examples of the preferred peptides of the present invention include, but are not limited to:

AAAAAQRYGRELRRMSDEFVDSFKK (SEQ ID. NO:1),
AAAAAQRYGRELRRMSDAFVDSFKK (SEQ ID. NO:2),
AAAAAQRYGRELRRMSDLFVDSFKK (SEQ ID. NO:3),
AAAAAQRYGRELRRMSDMFVDSFKK (SEQ ID. NO:4),
AAAAAQRYGRELRRMSDEFVDSKK (SEQ ID. NO:5),
AAAAAQRYGRELRRMSDKFVDSFKK (SEQ ID. NO:6),
NLWAAQRYGRELRRMSDKFVD (SEQ ID. NO:7),
AAAAAQRYGRELRRMSDEFVR (SEQ ID. NO:8), and
AAQRYGRELRRMSDEFVR ((SEQ ID. NO:9).

The mutant peptides of the present invention have been found to bind tightly to other human Bcl-2 family proteins, such as Bcl-x_L and Bcl-2. Moreover, the mutant peptides of the present invention exhibit a high helix propensity and maintain the native contacts of the wild-type peptide with the Bcl-2 family protein to which they bind. In addition, the mutant peptides may have improved physical properties, specifically, solubility, compared to the wild-type human Bad peptides.

The mutant peptides of the present invention can be prepared using techniques known in the art. For example, a nucleotide sequence encoding amino acid residues 137 to 169 from the naturally occurring Bad protein can be treated with a chemical mutagen, such as a base analog (i.e., as 5-bromouracil), a deaminating agent, or an alkylating agent (i.e., ethyl methane sulfonate (EMS)), or with a physical mutagen, such as UV or ionizing radiation or heat, using techniques known in the art.

The mutant peptides of the present invention can also be produced by recombinant DNA techniques known in the art. For example, nucleotide sequences encoding a mutant peptide having the hereinbefore described amino acid sequence, can be inserted into a suitable DNA vector, such as a plasmid. More specifically, the nucleotide sequence can be inserted into a suitable DNA vector using techniques known in the art, including, but not limited to, blunt-ending or staggered-ending termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are described in Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y., (1989). Once inserted the nucleotide sequence is inserted into the DNA vector, the vector is used to transform a suitable host. The recombinant mutant peptide is produced in the host by expression. The transformed host can be either a prokaryotic or eukaryotic cell.

Alternatively, the mutant peptides of the present invention can be directly synthesized

using various solid-phase techniques (see Roberge, J.Y. et al., *Science*, 269:202-204 (1995)) and automated synthesis may be achieved, for example, using the Applied Biosystems 431 A Peptide Synthesizer.

Once the peptides of the present invention have been prepared, they may be substantially purified by preparative high performance liquid chromatography (see Crighton, T., *Proteins, Structures and Molecular Principles*, WH Freeman and Co., New York, N.Y. (1983)). The composition of any synthetic peptides of the present invention can be confirmed by amino acid analysis or sequencing (using the Edman degradation procedure). The mutant peptides of the present invention can be used in screening assays. More specifically, when fluorescently labeled, the mutant peptides of the present invention can be used to identify small molecules that induce or suppress apotosis in cells.

As discussed earlier, the mutant peptides of the present invention have the following amino acid sequence:

$(Xaa)_n$-Tyr-$(Xaa)_3$-Leu-$(Xaa)_4$-Asp-Xaa-Phe-Val-$(Xaa)_n$

where each n independently has a value of from 1 to 10 and where at least one of the Xaa amino acids is different from that of the wild-type Bad peptide. Based on the NMR-derived structure of the wild-type Bad peptide bound to Bcl-x_L (Fig. 2) and shown here for the first time, amino acid residues Tyr, Leu, Asp, Phe, and Val, in the above sequence, make key contacts with the protein and thus, are necessary for tight binding to a Bcl-2 family protein, such as Bcl-x_L or Bcl-2. Since the development of an effective screening assay depends on maintaining the tight binding of the peptide and the Bcl-2 protein, knowledge of these key contacts permits the rational design of labeled peptides which maintain high binding affinity, notwithstanding the added label. More specifically, in a preferred embodiment of the invention, a peptide is chemically modified (e.g. fluoresceinated) at one Xaa, i.e. at a non-contact residue, to maintain effective binding between the peptide and a Bcl-2 protein. In addition, since the amino terminus of the bound peptide does not contact the protein, it could also be modified without affecting binding affinity. All such labeled peptides are particularly useful in the competitive and/or displacement type assays

described below.

IV. Screening Assays Using the Peptides of the Present Invention

The present invention also relates to a variety of screening assays to identify candidate compounds that are capable of inducing or suppressing apoptosis in cells. The assays of the present invention focus on the ability or inability of candidate compounds to disrupt the binding interaction between the wild-type Bad peptide or a mutant Bad peptide of the present invention and a protein from the Bcl-2 family proteins, such as Bcl-x_L or Bcl-2. Preferred wild-type Bad peptides useful in the assays described herein have an amino acid sequence selected from the group consisting of:

NLWAAQRYGRELRRMSDEFVDSFKK (SEQ ID NO:10);
NLWAAQRYGRELRRMSDEFVDSFK (SEQ ID NO:27);
NLWAAQRYGRELRRMSDEFVDSF (SEQ ID NO:28);
NLWAAQRYGRELRRMSDEFVDS (SEQ ID NO:29); and
NLWAAQRYGRELRRMSDEFVD (SEQ ID NO:30)

The screening assays of the present invention can be used to screen large numbers of compounds to identify those compounds which are capable of inducing or suppressing apoptosis. For those compounds identified which induce apoptosis, these compounds can be used clinically for the treatment of certain cancers. For those compounds identified which are suppressors of apoptosis, these compounds can be used clinically for suppressing aberrant apoptosis in degenerative disorders or following ischemic injury. Compounds which do not have activity in the screening assays can be eliminated from further consideration as candidate compounds.

The candidate compounds to be screened can encompass numerous chemical classes. However, the candidate compounds are typically organic molecules, preferably small organic compounds having a molecular weight of from about 150 to about 800 daltons. Such candidate compounds shall contain functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or

carboxyl group, preferably at least two of the functional chemical groups. The candidate compounds often contain cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate compounds can also be found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate compounds can be obtained from a wide variety of sources such as libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs.

In competitive binding assays, the candidate compound can compete with a labeled analyte for specific binding sites on a binding agent bound to a solid surface. In such an assay, the labeled analyte can be the wild-type Bad peptide or a labeled mutant peptide derived from Bad and the binding agent can be any Bcl-2 family protein such as Bcl-x_L or Bcl-2 protein bound to a solid phase. Alternatively, the labeled analyte can be a labeled Bcl-2 family protein and the binding agent can be a solid phase mutant peptide derived from Bad. The concentration of labeled analyte bound to the capture agent is inversely proportional to the ability of the candidate compound to compete in the binding assay. The amount of inhibition of labeled analyte by the candidate compound depends on the binding assay conditions and on the concentrations of binding agent, labeled analyte, and candidate compound that are used. Under specified assay conditions, a candidate compound is said to be capable of inhibiting the binding of the wild-type Bad peptide or a mutant Bad peptide to a Bcl-2 family protein in a competitive binding assay, if the amount of

binding of the labeled analyte to the binding agent is decreased by ten percent (10%) or more. In a direct binding assay, a candidate compound is said to inhibit the binding of the mutant protein to a Bcl-2 family protein when the signal measured is twice the background level or higher.

In a competitive binding assay, the candidate compound competes with labeled peptides of the present invention for binding to a specific binding agent (i.e. a Bcl-2 family member). As described herein, the binding agent may be bound to a solid surface to effect separation of bound labeled protein from the unbound labeled peptides. Alternatively, the competitive binding may be conducted in a liquid phase, and any of a variety of techniques known in the art may be used to detect the release of the bound peptide or to separate the bound labeled peptides from the unbound labeled protein. Following separation, the amount of bound labeled peptides is determined. The amount of peptide present in the sample is inversely proportional to the amount of bound labeled peptide.

Alternatively, a homogenous binding assay can be performed in which a separation step is not needed. In these types of assays, binding of the labeled peptide to the protein results in a decrease or increase in the signal emitted by the label, thus allowing for quantification of the bound peptide.

An example of a competitive binding assay for detecting candidate compounds capable of inhibiting the binding of a wild-type Bad peptide or a mutant Bad peptide to a Bcl-2 family protein is described in Examples 1 and 2, herein. These examples describe a competitive fluorescence polarization assay where either the peptides or the synthetic compounds competed with a labeled mutant Bad peptide of the present invention for binding to Bcl-x_L.

As discussed hereinbefore, the screening assays described herein employ one or more labeled molecules. The label used in the assay of the present invention can directly or indirectly provide a detectable signal. Various labels that can be used include radioactive moieties, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds, enzymes,

specific binding molecules, particles, e.g., magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin, etc. For the specific binding members, the complementary member is normally labeled with a molecule that provides for detection, in accordance with known procedures. Furthermore, the binding of these labels to the mutant peptides of the present invention is accomplished using standard techniques known in the art. Fig. 2 shows some examples of the labeled peptides of the present invention. The peptides shown in this Figure are labeled with fluorescein isothiocyanate (FITC) or 5-carboxy-fluorescein (5'-FAM).

A variety of other reagents may also be included in the screening assay. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc. that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between about 0 and about 40°C. Incubation periods are selected for optimum activity. Typically, incubations from about 0.05 and 10 hours will be sufficient.

By way of example, and not of limitation, examples of the present invention shall now be given.

EXAMPLE 1: Competitive Binding Assays to Measure Peptide Affinities for Bcl-x_L

A competitive fluorescence polarization assay was used to measure the affinity of eighteen (18) peptides (Table 1) for Bcl-x_L using the fluorescein-labeled NLWAAQRYGRELRRMSDK(FITC)KFVD (Synpep Corporation, Dublin, CA) as a probe. The dissociation constant of this fluoresceinated peptide from Bcl-x_L is ~ 30 nM.

All titrations were automated by means of an Abbott clinical diagnostics instrument (IMx, FPIA mode) modified with a special protocol for performing titrations. A complete two-fold dilution

series, comprised of twenty separate 2 mL samples, was obtained by delivering appropriate individual aliquots to the first seven tubes, aliquots from an intermediate diluted stock for the next seven, and one more intermediate dilution for the final six. Dilution buffer for all stocks and samples was 120 mM sodium phosphate at pH 7.55 with 0.01% bovine gamma globulin and 0.1% sodium azide. The concentrations of the DMSO stock solutions of the peptide were 1-4 mM as determined by Trp absorbance (O.D. 280 nm), Tyr absorbance (O.D. 293), or amino acid analysis. The final DMSO concentration for all samples never exceeded 1%. Twenty 1.8 uL samples were prepared without flouresceinated peptide and read as blanks. To each tube, 0.2 uL of a Bcl-x_L, flouresceinated peptide mixture was added; the tubes were incubated for 5 min at 35° C, and then read for total intensity and polarization. Free and bound values for the flouresceinated peptide were constant within a range ± 5 mP. Final Bcl-x_L concentration was 114 nM. Comparisons with other, lower Bcl-x_L concentrations, were made for the wild type Bad peptide. Additional controls using buffer lacking BGG showed that non-specific binding to BGG was negligible.

Steady state polarization data can be analyzed to extract the fractions of bound and free fluorescent ligand owing to the linear additivity of their anisotropy values, weighted by their respective fractional intensities (Lakowicz, JR. *Principles of Fluorescence Spectroscopy.* New York, NY: Plenum Press (1983)). Nonlinear least squares curve fitting of titration data to a model for simple equilibrium binding of the fluoresceinated peptide to Bcl-x_L was accomplished by programming standard binding equations, solved in terms of bound, free, and observed anisotropy values, into the model development program MINSQ (V. 4.03, Micromath Scientific Software). To determine affinities of nonfluorescent peptides, the analytical approach for equilibrium competition binding taken by Dandliker and coworkers was used, again employing MINSQ for fitting of titration curves (Dandliker, et al., *Methods in Enzymology* 74: 3 – 28 (1981)). Confirmation of the validity of these experimental and fitting procedures was obtained by comparing results after performing fluorescenated peptide binding, and competition binding titrations at different fixed Bcl-xL or fluorescenated peptide concentrations.

Table 1:Peptide Binding to Bcl-x_L

Sequence		K_d(nM)
NLWAAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:10)	0.6
ALWAAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:11)	0.4
NAWAAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:12)	0.7
NLAAAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:13)	0.3
AAAAAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:14)	0.5
NLWGAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:14)	0.8
NLWAGQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:16)	2.4
NLWAAQRYGRELRRMSDEFVDAFKK	(SEQ ID NO:17)	0.3
NLWAAQRYGRELRRMSDEFVDSAKK	(SEQ ID NO:18)	2.1
NLWAAQRYGRELRRMSDEFVDSFAK	(SEQ ID NO:19)	1.2
NLWAAQRYGRELRRMSDEFVDSFKA	(SEQ ID NO:20)	0.2
GGGAAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:21)	0.2
NLPAAQRYGRELRRMSDEFVDSFKK	(SEQ ID NO:22)	0.3
NLWAAQRYARELRRMSDEFVAAFKK	(SEQ ID NO:23)	1.1
NLWAAQRYGREARRMSDEFVDSFKK	(SEQ ID NO:24)	656
NLWAAQRYGRELRRMSAEFVDSFKK	(SEQ ID NO:25)	19
QRYGRELRRMSDEFVDSFKK	(SEQ ID NO:26)	201
NLWAAQRYGRELRRMSDEFVD	(SEQ ID NO:9)	20

EXAMPLE 2: Competitive Binding Assays to Measure Affinities of Synthetic Compounds for Bcl-x_L

A competitive fluorescence polarization assay was used to measure the affinity of six (6) synthetic compounds shown in Fig. 3 for Bcl-x_L using the fluorescein-labeled mutant Bad peptide, NLWAAQRYGRELRRMSDK(fluorescein)FVD) as a probe. It was carried out in a 96-well format as follows. A stock solution of 5 nM of the above-described fluorescein-labeled mutant Bad peptide, 50 nM of for Bcl-x_L protein, 1 mM EDTA, and 0.05% PEG-8000 (polyethylene glycol) in 20 mM phosphate buffer, pH 7.4 was first prepared. For each compound shown in Fig. 3, 120 μl of this stock solution was placed into each of seven wells of a 96-well plate. To each well was added either 10, 3, 1, 0.3, 0.1, 0.03 or 0.01 μM compound form a DMSO stock. The total DMSO concentration in each well was then adjusted, if necessary, to 4% by the addition of neat DMSO. A negative control (5 nM Bad peptide, 4% DMSO in buffer) and positive control (5 nM Bad peptide, 50 nM Bcl-x_L, 4% DMSO in buffer) were used to determine the free and bound polarization values, respectively for the assay.

The 96 well plate was shaken for 2 minutes and the polarization was measured at room

temperature in a LJL Analyst (LJL BioSystems, CA) fluorescence plate reader with excitation at 485 nm and emission at 530 nm. IC_{50} values were obtained by non-linear least squares fitting of the data to the following equation:

Observed polarization = (bound polarization - free polarization)$1+10^{(LogIC50-Concentration)}$

IC_{50} values for the six compounds of Fig. 3 are provided below in Table 2.

Table 2: Binding of Synthetic Compounds to Bcl-x_L

Compound Number (See Fig. 3)	IC_{50} (nM)
1	85
2	293
3	427
4	601
5	1272
6	1720

These results show that a mutant Bad peptide can be used to evaluate the affinities of various synthetic compounds for Bcl-x_L and therefore suggest which compounds would make effective Bcl-x_L antagonists.

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

Changes can be made to the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims.

Any references referred to herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. An isolated and purified peptide derived from a wild-type human Bad peptide which binds to a member of the Bcl-2 family of proteins, said peptide having the amino acid sequence:

$(Xaa)_n$-Tyr-$(Xaa)_3$-Leu-$(Xaa)_4$-Asp-Xaa-Phe-Val-$(Xaa)_n$

where each n independently has a value of from 1 to 10; and

where at least one of the Xaa amino acids differs from that of the wild-type human Bad peptide.

2. The peptide of claim 1 wherein the Bcl-2 family of proteins is Bcl-xL.

3. The peptide of claim 1 wherein the Bcl-2 family of proteins is Bcl-2.

4. A peptide selected from the group consisting of:

AAAAAQRYGRELRRMSDEFVDSFKK (SEQ ID. NO:1),
AAAAAQRYGRELRRMSDAFVDSFKK (SEQ ID. NO:2),
AAAAAQRYGRELRRMSDLFVDSFKK (SEQ ID. NO:3),
AAAAAQRYGRELRRMSDMFVDSFKK (SEQ ID. NO:4),
AAAAAQRYGRELRRMSDEFVDSKK (SEQ ID. NO:5),
AAAAAQRYGRELRRMSDKFVDSFKK (SEQ ID. NO:6),
NLWAAQRYGRELRRMSDKFVD (SEQ ID. NO:7),
AAAAAQRYGRELRRMSDEFVR (SEQ ID. NO:8), and
AAQRYGRELRRMSDEFVR ((SEQ ID. NO:9).

5. An assay for identifying substances which bind to a member of the Bcl-2 family of proteins, the assay comprising the steps of:

(a) providing a candidate substance to be tested;

(b) forming a reaction mixture by contacting the candidate substance with a Bcl-2 family protein and a labeled peptide having the amino acid sequence:

$(Xaa)_n$-Tyr-$(Xaa)_3$-Leu-$(Xaa)_4$-Asp-Xaa-Phe-Val-$(Xaa)_n$

where each n independently has a value from 1 to 10;

(c) incubating the reaction mixture under conditions sufficient to allow the candidate substance to react and bind with the Bcl-2 family protein; and

(d) determining whether the candidate substance has bound to the Bcl-2 family protein by determining whether the labeled Bad peptide has been displaced from binding to the Bcl-2 family protein.

6. The assay of claim 5 wherein the peptide is labeled with a radioactive moiety, a fluorescent moiety, an enzyme, or a specific binding molecule or particle.

7. The assay of claim 6 wherein the peptide is labeled with a fluorescein compound.

8. The assay of claim 7 wherein the peptide is labeled with fluorescein isothiocyanate or 5-carboxy-fluorescein.

9. The assay of claim 5 wherein the peptide is selected from the group consisting of:

AAAAAQRYGRELRRMSDEFVDSFKK (SEQ ID. NO:1),
AAAAAQRYGRELRRMSDAFVDSFKK (SEQ ID. NO:2),
AAAAAQRYGRELRRMSDLFVDSFKK (SEQ ID. NO:3),
AAAAAQRYGRELRRMSDMFVDSFKK (SEQ ID. NO:4),
AAAAAQRYGRELRRMSDEFVDSKK (SEQ ID. NO:5),
AAAAAQRYGRELRRMSDKFVDSFKK (SEQ ID. NO:6),
NLWAAQRYGRELRRMSDKFVD (SEQ ID. NO:7),
AAAAAQRYGRELRRMSDEFVR (SEQ ID. NO:8), and
AAQRYGRELRRMSDEFVR ((SEQ ID. NO:9).

10. The assay of claim 5 wherein the Bcl-2 family protein is Bcl-xL.

11. The assay of claim 5 wherein the Bcl-2 family protein is Bcl-2.

12. The assay of claim 5 wherein said amino acid sequence of said labeled peptide has at least one Xaa amino acid that is different from that of the wild-type Bad peptide.

13. The assay of claim 12 wherein the peptide is labeled with a radioactive moiety, a fluorescent moiety, an enzyme, or a specific binding molecule or particle.

14. The assay of claim 13 wherein the peptide is labeled with a fluorescein compound.

15. The assay of claim 14 wherein the peptide is labeled with fluorescein isothiocyanate or 5-carboxy-fluorescein.

16. The assay of claim 12 wherein the Bcl-2 family protein is Bcl-xL.

17. The assay of claim 12 wherein the Bcl-2 family protein is Bcl-2.

18. The assay of claim 5 wherein the peptide is selected from the group consisting of

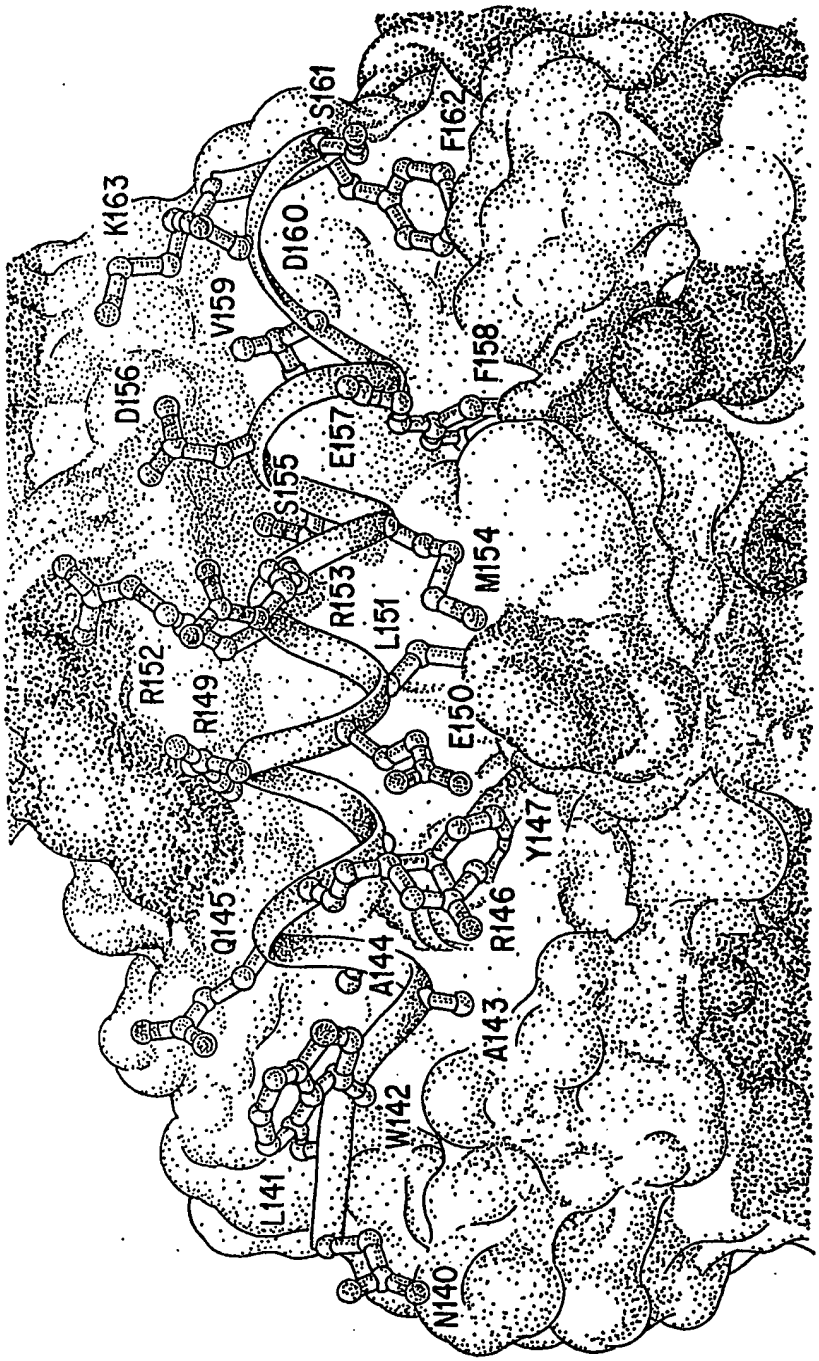
(a) NLWAAQRYGRELRRMSDEFVDSFKK (SEQ ID NO:10);

(b) NLWAAQRYGRELRRMSDEFVDSFK (SEQ ID NO:27);

(c) NLWAAQRYGRELRRMSDEFVDSF (SEQ ID NO:28);

(d) NLWAAQRYGRELRRMSDEFVDS (SEQ ID NO:29); and

(e) NLWAAQRYGRELRRMSDEFVD (SEQ ID NO:30).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Q145
R146
A144
A143
W142
L141
N140

FIG. 1

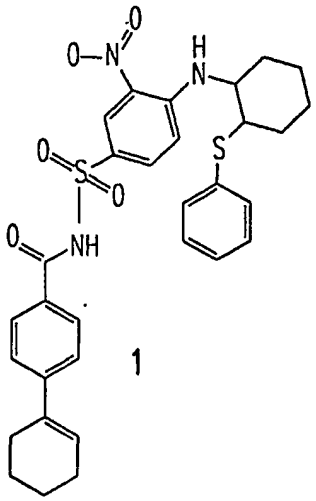
(5-FAM)-AAAAAQRYGRELRRMSDEFVDSFKK
(5-FAM)-AAAAAQRYGRELRRMSDAFVDSFKK
(5-FAM)-AAAAAQRYGRELRRMSDLFVDSFKK
(5-FAM)-AAAAAQRYGRELRRMSDMFVDSFKK
(5-FAM)-AAAAAQRYGRELRRMSDEFVDSKK

(5-FAM)
|
AAAAAQRYGRELRRMSDKFVDSFKK

(FITC)
|
NLWAAQRYGRELRRMSDKFVD

(FITC)-AAAAAQRYGRELRRMSDEFVR
(FITC)-AAQRYGRELRRMSDEFVR

FIG.2



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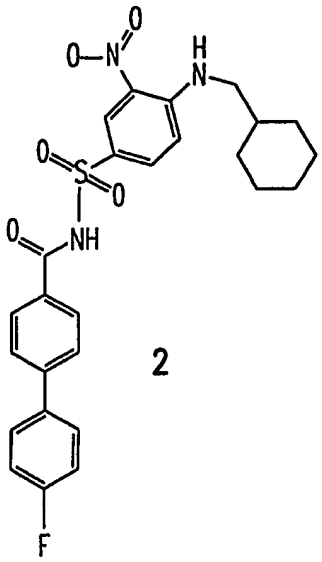

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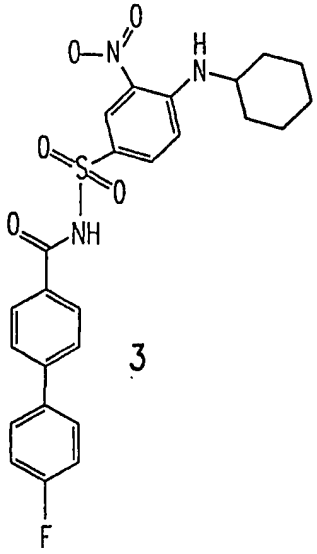

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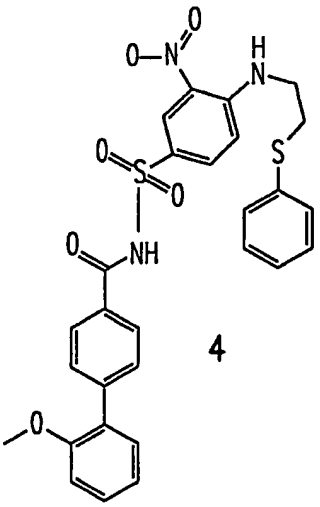

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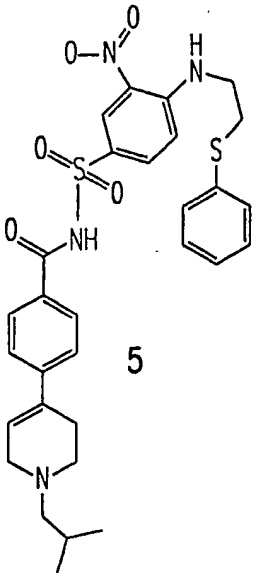

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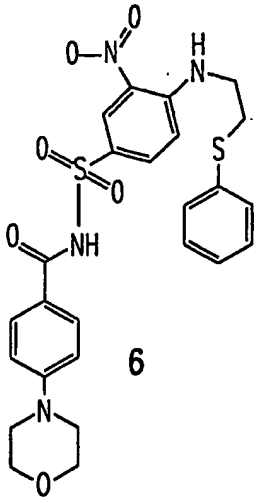

6

FIG.3

SEQUENCE LISTING

<110> Fesik, Stephen F.
Meadows, Robert P.
Joseph, Mary K.
Oleijiczak, Edward T.
Petros, Andrew M.
Nettesheim, David G.
Swift, Kerry M.
Matayoshi, Edmund
Zhang, Haichao

<120> MUTANT PEPTIDES DERIVED FROM BAD AND
THEIR USE TO IDENTIFY SUBSTANCES WHICH BIND TO A MEMBER OF
THE BCL-2 FAMILY OF PROTEINS

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